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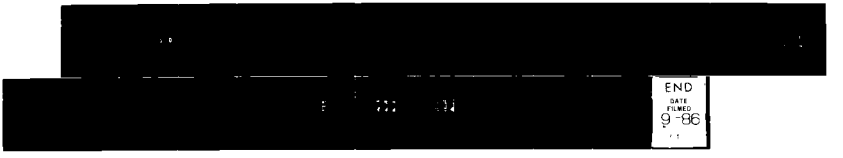

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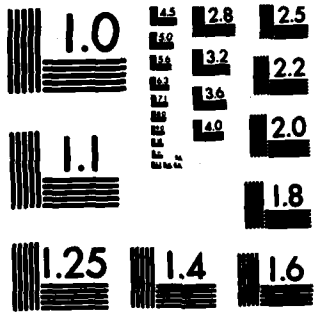

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SYNTHETIC PEPTIDE VACCINES FOR THE CONTROL OF ARENAVIRUS INFECTIONS

ANNUAL REPORT

M. J. BUCHMEIER and PETER SOUTHERN
SCRIPPS CLINIC AND RESEARCH FOUNDATION

SEPTEMBER 1983

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

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Scripps Clinic and Research Foundation
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Arenaviruses are endemic on both the African and South American continents and represent significant health hazards. Precise diagnostic methods and effective treatment protocols are not currently available. We are using genetic cloning methods to develop an effective vaccine against arenaviruses. Developmental studies are being carried out and techniques established with the prototype arenavirus lymphocytic choriomeningitis virus (LCMV), and these techniques will be applied for rapid future development of vaccines against

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the pathogenic arenaviruses Lassa, Junin and Machupo. Using techniques of protein chemistry we are identifying and mapping important immunogenic regions within the LCMV glycoproteins. The LCMV genomic RNAs have been cloned and the primary sequence of the viral polypeptides deduced from the cloned viral cDNAs. Short peptides corresponding to the immunogenic regions will be synthesized chemically and assayed for ability to induce protective neutralizing antibody responses in experimental animals. Experimental approaches to therapy for acute arenavirus infections will also be explored. These will include using cloned cytotoxic T-lymphocytes and antisera against components of the major histocompatibility complex (H-2 in mice) in attempts to modify the progress of acute disease in mice.

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SUMMARY

In our original research Contract C3013 entitled "Synthetic peptide vaccines for control of arenavirus infection" we proposed to develop and utilize synthetic peptide vaccines to control arenavirus infection. In addition, we plan to investigate the potential of immune reagents to control and modify ongoing arenavirus infections in animals. The first year of the proposal has focused on cDNA cloning, sequencing and developing synthetic peptides from lymphocytic choriomeningitis virus (LCMV). In subsequent years we will be concerned with testing these reagents for their ability to control LCMV infection in animals. LCMV will serve as a model system for work on other arenaviruses (Machupo, Junin, Lassa). The experimental approach involves transcribing arenavirus RNA segments into cDNA, cloning the cDNA, determining the nucleotide sequence and deducing the amino acid sequences for the viral polypeptides. The amino acid sequence is then inspected to locate regions that may represent antigenic determinants. Previous work done at this Institute with synthetic peptides for influenza virus hemagglutinin and hepatitis B virus will serve as a guide for our construction of arenavirus peptides. The technique involves synthesizing peptides of 10-20 residues in length containing at least one proline and made up primarily of hydrophilic amino acids. Such measures generally result in the selection of peptides capable of inducing antibodies. We will chemically synthesize LCMV specific peptides (of about 10-20 amino acids), use these peptides to raise immune responses, and test those responses in both mouse and guinea pig models for protective effect. It is hoped that with the technology developed here, on LCMV and using RNA from Machupo, Junin and Lassa Fever viruses provided by investigators at Fort Detrick, we will be able to develop similar reagents for the control of Machupo, Junin and Lassa Fever virus infections. Specific peptide vaccines developed from these arenaviruses would be tested for vaccine potential in primates at Ft. Detrick.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

The investigator(s) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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II. Summary Progress Report - year 01

A. Introduction

The results described in the following summary progress report cover the first 9 months of work on this contract (1 December 1982 through 31 August 1983). The initial contract period covers 15 months from 1 December 1982 through 28 February 1984. During the first 9 months we have focused on two primary goals as outlined in the original research proposal and this report will be divided accordingly. These goals were 1) cDNA cloning and sequencing of the lymphocytic choriomeningitis virus (LCMV) RNA segments focusing particularly on the S RNA segment and 2) identifying and mapping antigenic determinants on the LCMV glycoproteins. In addition to this we have done comparative peptide mapping on Lassa, Mozambique and ACAR virus NP and GP-2 proteins supplied to us by CDC in order to begin to evaluate the degree of homology among these viruses.

B. Cloning and sequencing of LCMV RNAs

The genome of LCMV comprises two segments of single stranded RNA with lengths approximately 9kb and 4kb (RNAs designated L and S, respectively) (1). The purified viral RNAs are non-infectious and, on this basis, the RNAs have been assigned a negative polarity (2). The strategy adopted for cDNA cloning was designed to take account of two particular problems: a) the negative polarity of the virion RNAs, i.e., the absence of a poly A tract at the 3' ends of the virion RNAs and b) contamination of viral RNA preparations with host ribosomal RNAs.

We used the enzyme polynucleotide phosphorylase (3) to add a poly A tail to the 3' ends of LCMV virion RNA preparations and isolated the poly A^+ RNA fraction by retention on oligo dT cellulose (4). A synthetic dT primer (dT 8-12 Collaborative Research) was annealed to the poly A^+ virion RNAs to initiate reverse transcription of the RNA templates (i.e., cDNA synthesis). The extent of cDNA synthesis was monitored by including a trace of $\gamma^{32}P$ dCTP in the reverse transcription reaction and following the TCA insoluble radioactivity. The LCMV RNA samples were denatured by heating to 70°C for 5 minutes immediately prior to addition to the reverse transcription reactions but, even after this treatment, it is likely that extensive regions of secondary structure would re-form and this could contribute to the comparatively short lengths of the cDNAs that have been cloned.

The products of reverse transcription reactions (RNA-DNA duplexes) were treated with alkali (0.1N NaOH, 10 hours, 37°C) to degrade the RNA and then the DNA strands were purified on a G-50 Sephadex gel-filtration column. The second DNA strand was synthesized with Klenow DNA polymerase I in a reaction that required looping back on the first strand to create a region of duplex which functioned as a primer for the polymerase reaction (5). The double-stranded cDNAs were then treated with S1 nuclease to remove the loop structure and also to remove any single-stranded regions remaining because of incomplete synthesis by DNA polymerase I.

The LCMV cDNA segments were cloned into the Pst I restriction endonuclease recognition site of the bacterial plasmid pBR322 using the homopolymer tailing technique (6). With the enzyme terminal transferase, 15-25 nucleotides of dC or dG were added to the cDNA and the pBR322, respectively, and after annealing to allow G:C base pairing, mixtures of these DNAs were used to transform *E. coli* HB101 cells. Potential recombinant plasmids (containing cDNA inserts) were identified on the basis of antiobiotic resistance properties of the *E. coli* cells (pBR322 confers resistance to both ampicillin and tetracycline, recom-

binant plasmids with insertions at the Pst I site produce ampicillin sensitive, tetracycline resistant cells). The rationale for the G:C tailing was based on the observation (7) that the Pst I site in pBR322 should be precisely reconstructed and this would provide a very simple method to separate the cDNA inserts from the plasmid vector. So far, more than 90% of the cDNA clones have Pst I sites at both the junctions between the plasmid and cDNA sequences.

Bacterial colonies that contained LCMV cDNA inserts were identified by colony hybridization (8). The probes for this hybridization screening were prepared from purified LCMV virion RNA - the RNA was fractionated by agarose gel electrophoresis and the LCMV L and S bands were excised from the gel and the RNAs were recovered, these RNA samples were then mildly degraded with alkali and labelled at the 5' ends with γ^{32}P ATP and polynucleotide kinase (9). The nitrocellulose filters from the colony hybridization were hybridized sequentially with L and S probes and a number of positive colonies were detected with each probe (Fig. 1).(Subsequent analysis has shown that there is a good correlation between the intensity of the hybridization signal and the sizes of the inserted cDNA fragments).

We have focused our attention on the S cDNA clones because of the known location of the viral glycoprotein coding region within the S RNA segment. A number of clones have been characterized as containing sequences from the 3' portion of the S RNA (see Fig. 2) and the overlapping regions have been confirmed by restriction mapping and DNA hybridization studies. Several S cDNA clones have not yet been positioned in this diagram but unfortunately none of these clones overlaps to provide continued progression in 3' to 5' direction. In this coming year, we will be exploring methods to complete the cloning of the S RNA segment.

We have started to sequence the S cDNA clones and have determined the sequence of 620 bases from the 3' end of the S virion RNA. The nucleotide sequence information has predicted a protein coding region that remains open to the end of the known sequence and contains 120 amino acid residues. By analogy with other arenaviruses (D.H.L. Bishop, W-C Leung both unpublished), this is likely to be the nucleocapsid coding region although we will design experiments to verify this assignment. The nucleotide sequencing will progress in parallel with the cloning experiments and very shortly we should have 1200-1500 bases of S RNA sequence (see Fig. 2).

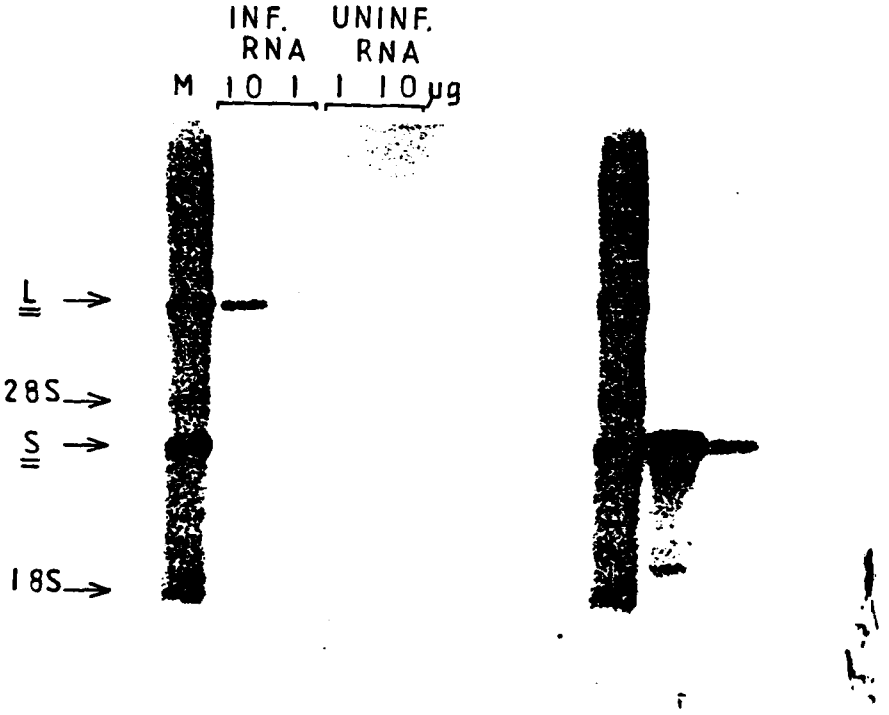
C. Analysis of antigenic determinants of the LCMV glycoproteins

During the first 9 months of this contract we have focused on definition of antigenic determinants of the LCMV glycoproteins and assessing both their variability among Old World arenaviruses and their biological activities. We initially raised a library of 23 monoclonals to the LCMV glycoproteins GP-1 and GP-2. The reactivity of this panel of 23 antibodies was assessed by immunofluorescence and ELISA assays against 6 commonly used laboratory strains of LCMV (CA1371, WE, UBC, Traub, Pasteur Inst. and 325a) as well as Lassa and Mozambique spot slides kindly supplied by Dr. P. Jahrling (USAMRIID). Three patterns of reactivity have emerged (Table 1). One group consisting of 9 antibodies, including 4 of confirmed anti GP-1 reactivity, bind to all of the 6 laboratory strains of LCMV but do not react with the African arenaviruses. These 9 antibodies functionally define framework antigens of LCMV which are highly conserved among strains. A second group of 8 antibodies react in a strain dependent fashion with specific viruses in the LCMV panel. That is, these antibodies react with one or more of the 6 LCMV strains, but none react with all strains. We intend to exploit these antibodies to define and localize genetic differences

FIGURE 1

Detection of LCMV RNAs by hybridization with radioactively labelled cDNA probes

Total cellular RNA was extracted from LCMV infected BHK cells and from control, uninfected cells. The RNAs were denatured with glyoxal, fractionated on the basis of size by agarose gel electrophoresis, transferred to nitrocellulose and then hybridized with ^{32}P cDNA probes that had been labelled by nick translation. The left panel shows the hybridization pattern with an L specific probe and then the filter was washed extensively and re-hybridized with an S specific probe (right panel). Note that neither the L nor S probe hybridizes to uninfected cell RNA and that there is no apparent homology between the L and S RNAs shown by this pair of probes. The track labelled M represents a sample of *in vivo* labelled LCMV virion RNA and shows ribosomal 28S and 18S and LCMV L and S RNA bands as markers.



among LCMV strains. The third group consists of 6 anti GP-2 antibodies. All of these 6 monoclonals reacted in immunofluorescence with all of the LCMV strains examined, but unlike the two groups described above, 5 of these also reacted with Lassa or Mozambique or both thus providing additional evidence of conservation of genetic information coding for GP-2 among LCMV, Lassa and Mozambique viruses. Three of these 5 antibodies (4BWE-33.6, 4BWE-83.5 and 4BWE-57.6) also reacted strongly in ELISA assays against purified Pichinde virus suggesting that a portion of GP-2 may be conserved between the New and Old World arenaviruses as well.

These observations are important to the contractural studies for two reasons; 1) because they allow a definition of LCMV isolates on the basis of specific antigenic determinants and provide "probes" in the form of monoclonals which can be exploited to map and isolate these unique sites, and 2) demonstration of a significant number of anti GP-2 antibodies cross reacting with the African viruses, as well as the previous identification of 13 anti LCMV nucleocapsid monoclonal antibodies which react with these agents (Buchmeier, unpublished data, 1983), presents compelling evidence of their close evolutionary link with LCMV, and will allow mapping and eventual molecular comparison of these shared regions on GP-2 and NP.

We have identified three monoclonal antibodies among our panel of antiglycoprotein antibodies which show strong virus neutralizing activity in the absence of complement. In assays against the Armstrong (CA1371) and WE strains of LCMV (Table 2), we find that one of the three antibodies (clone 2WE-6.2) neutralizes both strains of virus (corresponding to previous results by ELISA and immunofluorescence assays) indicating a framework reactivity. Two other antibodies, 2-11.10 and 4BWE-40 selectively react with the CA1371 and WE strains respectively, but show no interstrain cross reactivity. These results are of importance because they define a region(s) of the GP-1 molecule which serves as a target for virus neutralization. We will use these antibodies to identify and specifically isolate that region during the next project period.

In pursuing this further, we have prepared solid phase immunoadsorbants from these antibodies by binding them to CNBr activated Sepharose 4B and begun to develop methodology to affinity purify GP-1 from each virus. Briefly, radiolabeled purified virions are disrupted in 2% Triton x 100 and 0.5 M NaCl in phosphate buffer pH 7.2 then applied to a 4 ml column (with approximately 1-3 mg of purified IgG bound per ml of beads). The column is extensively washed with 0.1% Triton X 100 + 0.5 M NaCl to remove unbound protein then detergents are exchanged by further washing with 10 mM octyl-β, D-gluoside to replace the triton with a dialysable detergent. Bound glycoprotein is then eluted with 0.1 M di ethylamine (pH 11.5) in water and the fractions containing the eluted glycoprotein are pooled and lyophilized. Using this methodology we have been able to recover limited quantities of NP, GP-1 and GP-2 and are currently scaling up the procedure to supply sufficient quantities of these proteins for biochemical and immunochemical analyses during the next quarter.

In addition to the immunoaffinity chromatography approach we have also purified structural proteins of LCMV by preparative SDS-PAGE. Briefly, 400-600 µg of LCM virus is dissociated in 2% SDS and 1% β-mercaptoethanol then loaded onto a 10.5% gel in a single 11 cm wide sample slot and electrophoresed. Following electrophoresis the gel is stained to localize the LCMV proteins and the stained bands are cut out with a razor and homogenized into 0.1% SDS in water. Protein is extracted from the gel by rotating end-over-end overnight in a screw cap tube and recovered by precipitation with 10 volumes of absolute ethanol overnight at -20°C. The precipitated protein is washed once with 90% ethanol then dissolved in 1 ml of water and lyophilized to remove residual

FIGURE 2

Diagrammatic representation of overlapping clones extending from the 3' end of the LCMV S virion RNA

These overlaps were established by DNA-DNA hybridization (Southern Blotting) experiments. Clone S9 is lacking only 6 bases from the 3' end of the virion S RNA as inferred from direct RNA sequencing experiments (17). Regions covered by our nucleotide sequencing studies to date are indicated and gaps in the sequence are shown by the dashed lines. The diagram also shows the translation initiation codor for the major predicted protein coding sequence.

Clone S9 was isolated from 3' poly A addition and dT primed cloning reactions whereas the other clones were derived from random primed reverse transcription reactions.

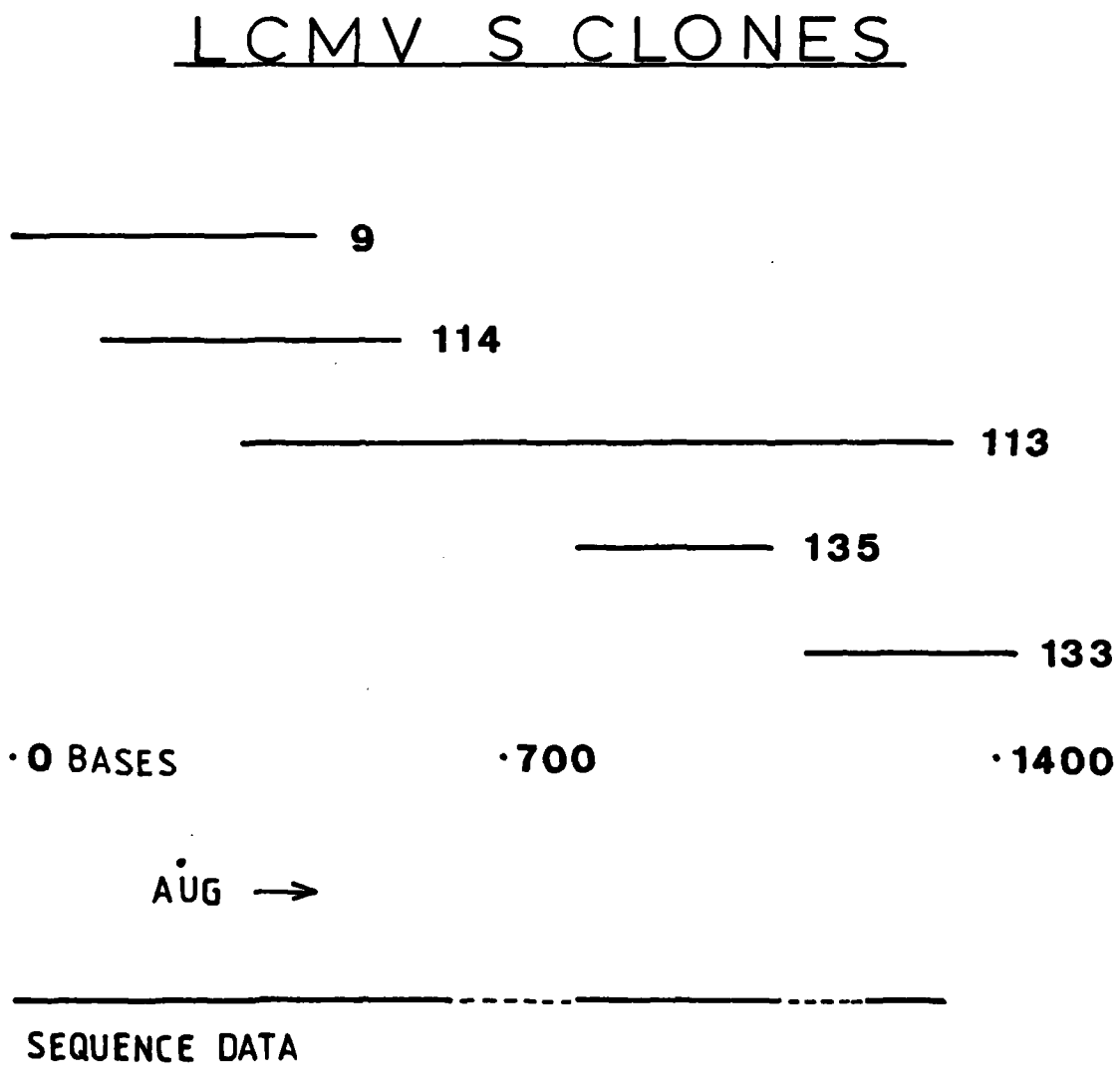


Table 1. Summary of hybridoma cross reactivity among Old World Arenaviruses

Hybridoma Antibody	Polypeptide Specificity	Reaction with LCMV Strain						African Arenaviruses	
		CA1371	WE	UBC	TRAUB	PASTEUR	325a	Lassa	Mozambique
2WE-2	GP-1	+	+		+	+	+	-	-
2WE-6.2	GP-1	+	+	+	+	+	+	-	-
4BWE-67	GP-1	+	+	+	+	+	+	-	-
4AWE-18	GP-1	+	+	+	+	+	+	-	-
1AWE-7.5	GP	+	+		+	+	+	-	-
4AWE-103	GP	+	+	+	+	+	+	-	-
5AWE-2	GP	+	+		+	+	+	-	-
23A-6.15	GP	+	+		+	+	+	-	-
26-20.8	GP	+	+		+	+	+	-	-
4B-WE-28	GP-2	+	+	+	+	+	+	-	-
4BWE-83.6	GP-2	+	+	+	+	+	+	+	+
4BWE-33.6	GP-2	+	+	+	+	+	+	+	+
4BWE-57.6	GP-2	+	+	+	+	+	+	+	+
4BWE-11.4	GP-2	+	+	+	+	+	+	-	+
9-7.9	GP-2	+	+	+	+	+	+	-	+
2-11.10	GP-1	+	-	-	-	-	-	-	-
5BWE-24	GP	+	+	+	+	-	+	-	-
4BWE-36	GP	+	+	-	+	+	-	-	-
4BWE-60	GP	+	+	-	+	+	-	-	-
4AWE-40	GP	-	+	-	-	+	-	-	-
1-16.3	GP	+	-	-	-	+	-	-	-
2-8.1	GP	+	-	-	-	-	-	-	-
1AWE-1.2	GP	-	+	-	-	-	+	-	-

ethanol. Gel analysis of the product shows a single band of protein. While this procedure clearly results in protein denaturation, samples prepared in this manner will be suitable for microsequencing and peptide cleavage experiments.

As mentioned above, we have developed an ELISA assay for antiviral antibody which is both sensitive and specific. For analysis of antibody specificity the test is performed as follows: Target plates are prepared by binding 0.1 to 1.0 µg of purified virus in bicarbonate buffer to each well of polycarbonate microtiter plates. Following overnight antigen binding, the plates are saturated by filling each well with tissue culture medium containing 5% fetal calf serum for 60 min at 22°C. For the assay, fourfold dilutions of monoclonal antibody diluted in PBS + 0.1% Tween 20 and 1% FCS are placed in the antigen wells and incubated 60-75 min at room temperature. Following incubation, plates are washed 3 times with 0.1% Tween 20 in PBS then incubated with horseradish peroxidase conjugated goat antibody to mouse IgG (1/2000 diln.) for an additional 60 min. Following this second antibody incubation, the plates are again washed 3 times then reacted with orthophenylenediamine substrate for 20 min. After stopping the reaction the OD at 492 nm is read using a Titertek multiscan spectrophotometer. Using this assay we have found that many of our monoclonal ascites preparations exhibit titers in excess of 1.6×10^6 against LCMV virion antigen. Critical factors which determine the sensitivity of the assay are antigen concentration in the wells, NP being readily detectable at a lower concentration than GP, and antibody avidity. Using this assay we have been able to determine quantitatively the endpoint titers of our monoclonals against the a panel of 4 LCMV strains. The test will be adapted to yield avidity measurements which will be of importance in evaluating antipeptide antibodies. In addition, this ELISA assay will form the basic test which we will use to monitor purification of peptides isolated from the viral glycoproteins.

We have recently been supplied by Dr. Michael Kiley of the Centers for Disease Control with protein samples of Lassa, Mozambique and ACAR (Arenavirus of the Central African Republic; 10) viruses for purposes of peptide mapping analysis. We were able to isolate NP and GP-2 from the gel samples we received (Fig. 3) and have prepared ^{125}I tryptic peptide maps of each protein (Fig. 4, 5). The results of this analysis demonstrate that 1) all of these three agents share several common tryptic peptides in the NP and GP-2 polypeptides, and 2) more homology is seen between the ACAR and Mozambique viruses than between these agents and Lassa. This data is entirely consistent with the concept that these viruses are related but distinct agents. To extend this analysis, we hope to obtain material suitable for antigenic analysis with both monoclonal and antipeptide antibodies during the next project period.

Recently we have performed a pilot experiment attempting to alter the course of LCMV induced disease in mice by passive administration of monoclonal ascites (Table 3). Four groups of 8 mice each received 3 different anti GP (2WE-6.2, 26-20.8, 23A6.12) antibodies and one received an anti NP ascites (25-22) one day prior to intracerebral challenge with 1000 pfu of LCMV Armstrong. A control group received no antibody. The mice were observed for classical symptoms of LCM disease and mortality scored over a 3 week period. One antibody, 2WE-6.2, extended the life of infected mice from 6.7 days (mean death day) to 16.5 days, and 3 of the infected mice survived the full 21 day duration of the experiment. Although the results are quite preliminary they suggest that the neutralizing antibody 2WE6.2 confirs some protection against disease <u>in vivo</u> as well. Further studies of the mechanism of this protection, effect of <u>antibody</u> on virus replication and immune responses will be done during the next project period.

Table 2. Summary of neutralization of LCMV strains by monoclonal antibodies

Monoclonal Antibody	Specificity	PRD_{50} Titer vs. LCMV[1]	
		Armstrong (CA1371-83.1)	WE
2WE-6.2	GP-1	525	1778
2-11.10	GP-1	> 10,000	< 20
4AWE-40	GP-1	< 20	562
9-7.9	GP-2	< 50	< 50

[1]Dilution of antibody giving 50% reduction of ~100 pfu of virus.

FIGURE 3

SDS polyacrylamide gel separation of Lassa (LAS) Mozambique strain MOPEA (MOP) and Arenavirus of the Central African Republic strain MOBALA (MOB). Gel material supplied by Dr. Michael Kiley, CDC, which shows N and G2 protetins which were analysed by tryptic peptide mapping as shown in the following figures.

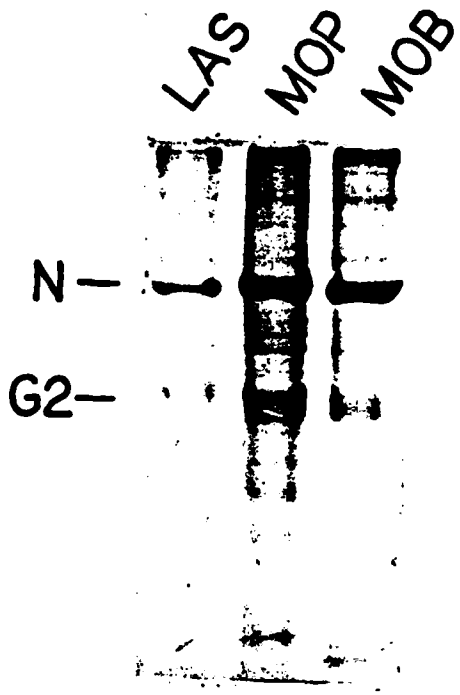


FIGURE 4

^{125}I tryptic peptide maps of the nucleocapsid (N) proteins of Lassa (LAS) Mozambique (MOP) and ACAR (MOB) viruses. The top row illustrates maps of the individual N proteins of each virus while the bottom shows the results of comigration of all of the combinations of digests. Note that MOP and MOB closely resemble each other and that two peptides (arrows) are apparently conserved among all of the viruses.

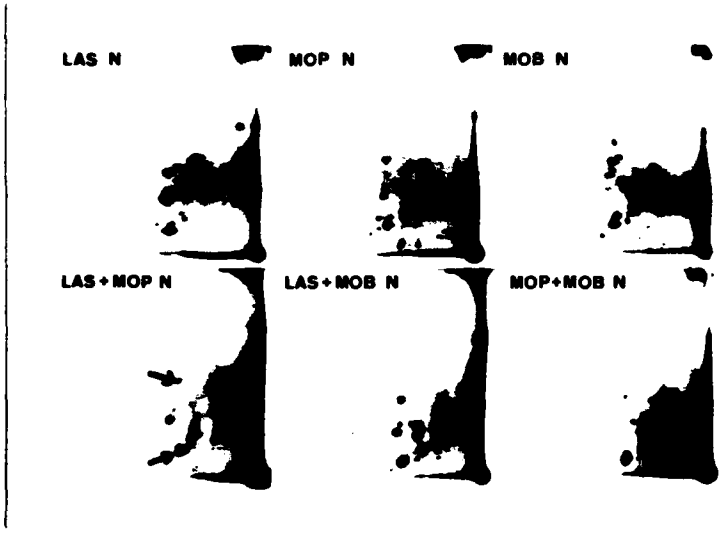


FIGURE 5

^{125}I tryptic peptide maps of the GP-2 glycoproteins (G2) of Lassa (LAS) Mozambique (MOP) and ACAR (MOB) viruses. The top row illustrates maps of the individual GP-2 proteins of each virus while the bottom shows the results of comigration of all of the combinations of digests. Note that MOP and MOB share several peptides in common as indicated by comigration in the MOB + MOP mixture.

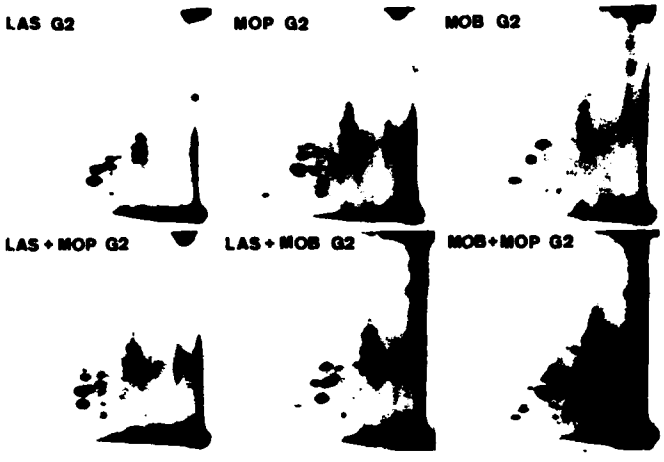


Table 3. Passive protection by monoclonal antibody of BALB/c mice against intracerebral challenge with LCMV-Armstrong

Monoclonal Antibody (d-1)	50% Survival on Day[1,2] (N=8 mice/group)
2WE-6.2	16.5
26-20.8	6.7
23A6.12	6.7
25-22	6.7
Control No Ab	6.7

[1] 200 µl of monoclonal ascites fluid given intraperitoneally on day -1 followed by 1000 pfu of LCMV ARM (CA1371 on day 0)

[2] Day on which 50% of mice were surviving interpolated from mortality curves

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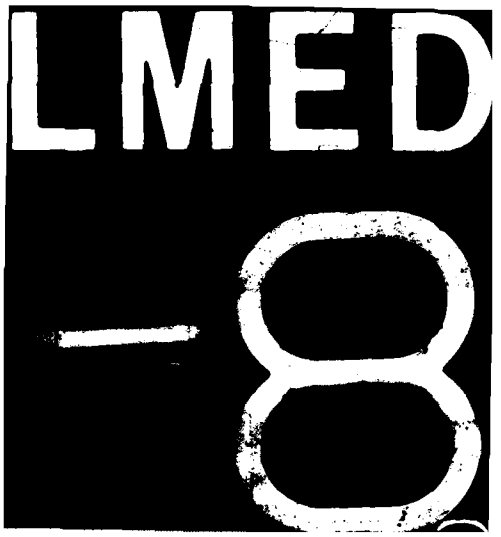
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